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14. ABSTRACT

About one-third of patients with cancer have metastases that are detected at the time their cancer is first diagnosed, and an additional third of patients have metastases that are too small to be detected by usual diagnostic tests. These micrometastases, however, will eventually grow into clinically significant metastases if the patient receives no treatment or local treatment of the primary tumor only. Metastatic breast cancer is a disease with low survival rates. The treatment of metastatic breast cancer involves the palliation of symptoms by reducing the tumor's size and growth rate. Currently, the effectiveness of therapy is determined by a series of tests. Imaging tests may include bone scan, radiograph, magnetic resonance imaging (MRI), and computed tomography (CT). Blood tests may include tumor markers, such as CA 27.29 and CA 15-3. The detection of circulating tumor cells has been proposed as a method to assess response to treatment of metastatic breast cancer. The detection of tumor cells may have clinical utility in risk stratification in early breast cancer, in early detection of relapse, and in monitoring the response to treatment. The circulating cells appear to have characteristics of tumor cells and may be identified in the peripheral blood of patients with cancer. The techniques that have been used to detect circulating tumor cells include cytometric and nucleic acid based approaches. The cytometric approaches use immunocytochemical methods to identify and characterize the individual tumor cells. Nucleic acid-based approaches detect the DNA and RNA sequences that are differentially expressed in tumor cells and normal blood components. The purpose of this study is to determine whether WAVE3, a metastasis promoter gene, can be used as a predictive marker for the progression and metastasis of breast cancer, using immunohistochemistry on human breast cancer tumors of different stages of breast cancer. An additional objective is to determine whether WAVE3 expression levels in the blood of breast cancer patients may have prognostic value after the administration of adjuvant chemotherapy in women with operable breast cancer. 14. ABSTRACT The first year of this study focused on identification and preliminary characterization of the tumor specimens and corresponding control samples. The Principal Investigator (PI) successfully identified most of the 100 tumor specimens (estrogen receptor [ER]-negative and histological grade III) with the most complete and informative clinical and pathological data from the Roswell Park Cancer Institute (RPCI) Tumor Registry. An additional cohort of 100 ER+ and histological grade I tumors is currently being identified.

15. SUBJECT TERMS
Breast cancer, Circulating Tumor Cells, WAVE3, Immunostaining, Metastasis

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Introduction

Cancer metastasis is a complex process, usually requiring cancer cells to escape from the primary site, survive in the blood/lymph system and then have the ability to establish at a distant site. We have shown that the expression levels of WAVE3, an actin polymerization protein, are significantly correlated with advanced stages of breast cancer, using immunohistochemistry analysis. Our preliminary data also suggest that the ER^- tumors, which are believed to be associated with a poor prognosis, show the highest levels of WAVE3 staining. Together with our published data on the role of WAVE3 in breast cancer progression and metastasis, we hypothesize that WAVE3 may function as a metastasis promoter gene.

On the other hand, breast cancer is considered a systemic disease because early tumor cell dissemination may occur even in patients with small tumors. The association between the presence of circulating tumor cells (CTCs) in the blood of patients with metastatic carcinoma and short survival is gaining increased support from the findings of several studies. Furthermore, the presence of CTCs after adjuvant chemotherapy has been associated with a poor clinical outcome in patients with early-stage breast cancer. Moreover, the detection of tumor cells-specific biomarkers in the blood before and after the adjuvant systemic treatment could help to identify those patients who may have a substantial clinical benefit from a 'secondary' adjuvant treatment before the occurrence of overt metastasis.
We, therefore, proposed the following specific Aims

Specific Aim 1: To test whether WAVE3 levels can be used as a predictive marker for the progression and metastasis of breast cancer.
Specific Aim 2: To evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells after the administration of adjuvant chemotherapy in women with operable breast cancer.

Body

Specific Aim 1: To test whether WAVE3 levels can be used as a predictive marker for the progression and metastasis of breast cancer.

1.A. Performance of Tasks proposed in the SOW

Task 1: Specimen identification

We identified the tumor specimens for which the most complete and informative clinical and pathological data is available.

Approximately 400 women undergo treatment for primary breast cancer each year at the RPCI Breast Center each year. A database of these patients has been maintained since 1995 by a professional data manager that contains pertinent clinical, pathological and treatment information on each patient. Recurrence and survival data is maintained by the RPCI Tumor Registry and can be linked to the breast cancer database. For this study, we were able to identify **64 out of 100** proposed women who are ER negative and histologic grade III based on the standard Scarff-Bloom-Richardson grading system. We were able to match them with **64 out of 100** proposed women who are ER+ and grade 1

Quality control was performed for all the tumors to determine their suitability for the slide preparation and immunohistochemical (IHC) staining.

Task 2: Slide preparation and staining were performed according to what was proposed in the SOW:

Slide preparation and staining: At least 4 slides will be prepared from each tumor, and the quality of slides will also be assessed and only the slides that pass the quality control, as judged by our expert pathologists, will be used for the subsequence staining procedures.

For each tumor we will perform the following stainings:

1) H&E to confirm the presence of tumor tissue and to determine the extent of tumor invasiveness.

2) Staining for the WAVE3 protein using a polyclonal rabbit anti-WAVE3 antibody, which has already been confirmed as suitable for IHC staining (Sossey-Alaoui et al., 2007).

3) Staining with rabbit IgG as negative control and to determine the back ground level of the staining.

Task 3: Tissue microarrays (TMA) preparation.

Since we were very satisfied with the quality of staining of the individual slides we determined that the TMA preparation has not become a high priority as least for the time being. We will however keep this task on the to do list.

Task 4: Scoring of the staining and Data analysis were conducted according to the SOW:

As stated above we have performed most of the Tasks for specific Aim 1 according to the time frame proposed in the SOW.

1.A. BACKGROUND

WAVE proteins coordinate actin cycling and cell motility.[1] The WAVE3 subtype is constitutively expressed in metastatic human breast cancer cell lines.[2] Its induction and activation results in membrane changes that enhance breast cancer cell migration.[2] Blockade of WAVE3 transcription disrupts breast cancer cell migration.[2] An association between WAVE3 over-expression and decreased estrogen receptor (ER) expression and histologic grade has been suggested.[3] WAVE3 expression is associated with enhanced breast cancer cell migration and adverse tumor features and, therefore, may be associated with the acquisition of metastatic potential in high risk tumors.

1.B. OBJECTIVE

The association between WAVE3 and ER status and histologic tumor grade was studied. WAVE3 expression and its association with the development of distant recurrence was also examined.

1.C. METHODS

Our institutional breast cancer (BC) database was reviewed for patients who presented with invasive BC from 1999-2009. A matched cohort design was utilized. Matching by pathologic stage and treatment was achieved for 61 patients with Scarff-Bloom-Richardson (SBR) grade 1 and ER+ tumors (SBR1/ER+) to 61 patients with SBR grade 3 and ER- tumors (SBR3/ER-). Cytosolic WAVE3 expression was determined by immunohistochemistry. The product of stain intensity (0-3) and percentage of cells staining (0-100) was used to derive a WAVE3 score (0-300). The WAVE3 score between each cohort was compared and the association between WAVE3 score and a variety of clinico-pathologic features was examined. The log rank test was used to compare distant recurrence free survival at various WAVE3 scores. A score of ≥212 was found to have the strongest association with distant recurrence and was used as a positive threshold for subsequent survival analyses.

Analysis of categorical data between two groups was performed with the χ^2 square test. Analysis of continuous variables for two groups was with the Mann-Whitney rank sum or the Pearson product moment correlation. Analysis of continuous variables for more than two groups was with the Kruskal-Wallis one way ANOVA . Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. The multiple linear regression method was performed to compare survival as a function of patient and treatment factors.

1.D. RESULTS

Table 1. Cohort clinical and tumor characteristics			
Variable	**SBR1/ER+ (n=61)**	**SBR3/ER- (n=61)**	**p**
Age at Diagnosis (years)	**57**	**57**	**0.479**
Tumor Size (cm)	**1.5**	**1.9**	**0.136**
Lymph Node Status			**0.714**
Negative	**34 (56%)**	**36 (59%)**	
Positive	**27 (44%)**	**25 (41%)**	
TNM Stage			**0.981**
Stage I	**22 (36%)**	**21 (34%)**	
Stage II	**31 (51%)**	**32 (53%)**	
Stage III	**8 (13%)**	**8 (13)%**	
Her2 Status			**<0.001**
Negative	**56 (92%)**	**41 (67%)**	
Positive	**3 (5%)**	**20 (33%)**	
Frequency of recurrence			**<0.001**
All recurrence	**1 (2%)**	**16 (26%)**	
Local recurrence	**1 (2)%**	**6 (10%)**	
Distant recurrence	**0 (0%)**	**10 (16%)**	
Disease specific mortality	**0 (0%)**	**7 (11%)**	**0.006**

Table 1. Increased Her-2 neu receptor expression, distant recurrence and breast cancer related mortality were seen in the SBR3/ER- cohort compared to the SBR1/ER+ cohort (33% vs 5%, p<0.001, 16% vs. 0%, p<0.001, and 11% vs. 0%, p=0.003, respectively).

Table 2. Association of WAVE3 score with ER status and SBR grade			
Variable	**SBR1/ER+ (n=61)**	**SBR3/ER- (n=61)**	**p**
Median WAVE3 score	**160**	**180**	**0.703**

Table 2. There was no difference in WAVE3 score between the two matched cohorts. (SBR1/ER+, 160 vs. SBR3/ER- ,180, p=0.703).

Table 3. Association between WAVE3 score and tumor features			
	Median WAVE3 score		
Variable	**All patients (n-122)**	**SBR1/ER+ (n=61)**	**SBR3/ER+ (n=61)**
Tumor size	**[a]0.234, p=0.009**	**0.201, p=0.120**	**[a]0.261, p=0.042**
Lymph Node Status			
Negative	**145**	**130**	**170**
Positive	**200**	**200**	**180**
TNM Stage			
Stage I	**160**	**140**	**200**
Stage II	**180**	**200**	**150**
Stage III	**240**	**190**	**255**
Her2 neu status			
Negative	**180**	**160**	**180**
Positive	**200**	**200**	**200**

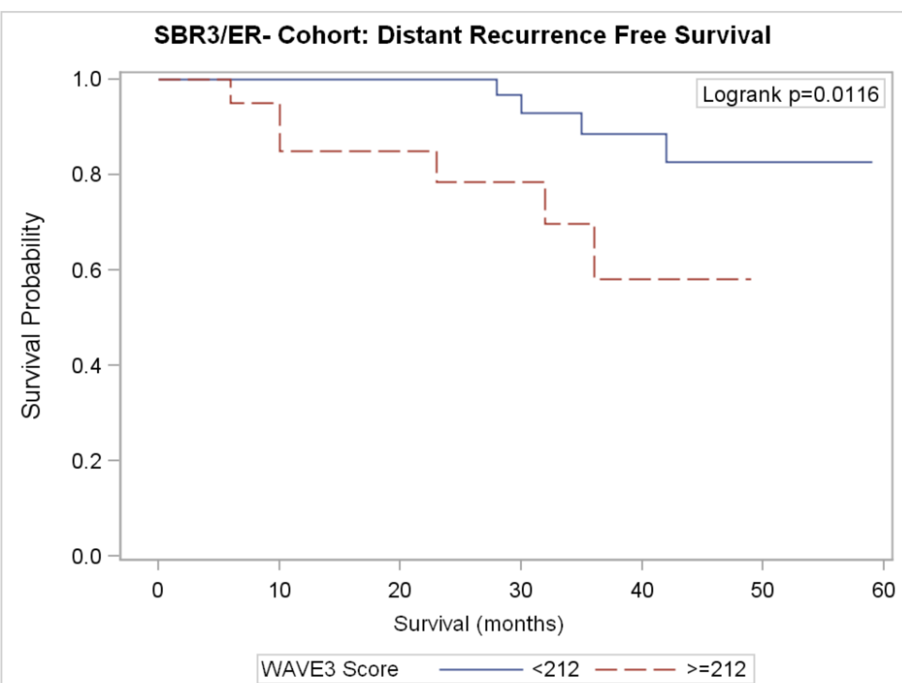
Table 3. Median WAVE3 score increased with tumor size for the entire study group (Pearson correlation 0.234, p=0.009), but only remained significantly associated for the SBR3/ER- cohort (Pearson correlation 0.261, p=0.042).
Median WAVE3 score increased with lymph node status (positive 200 vs. negative, 145, p =0.034), but only remained significantly associated for the SBR1/ER+ group (positive 130 vs negative 200, p=0.023).
Median WAVE3 score increased with pathologic stage (I, 160 vs. II,180 vs III, 240, p=0.012) but only remained significantly associated for the SBR3/ER- group (I, 200 vs II, 150 vs II, 255, p=0.006).
WAVE3 score and Her2-neu receptor status were not associated (Her2neu- 180 vs. Her2neu+ 200, p=0.509).

Table 4. Association between WAVE3 score and outcome			
	Median WAVE3 score		
Variable	All patients (n-122)	SBR1/ER+ (n=61)	SBR3/ER+ (n=61)
Distant recurrence			
No	NA	NA	160
Yes	NA	NA	240
Disease specific mortality			
No	NA	NA	170
Yes	NA	NA	270

Table 4. Only patients in the SBR3/ER- cohort experienced distant recurrence or disease specific mortality. WAVE3 scores were higher for patients with either adverse clinical outcome

1.B.1. Low staining of WAVE3 correlates with overall disease free survival.

We found a very significant correlation between the levels of WAVE3 staining in the primary tumors and the overall disease free survival. Those patients with low levels of WAVE3 staining in the primary tumors were found to live longer disease free after the primary tumors was removed compared to those patients with high levels of WAVE3 staining.



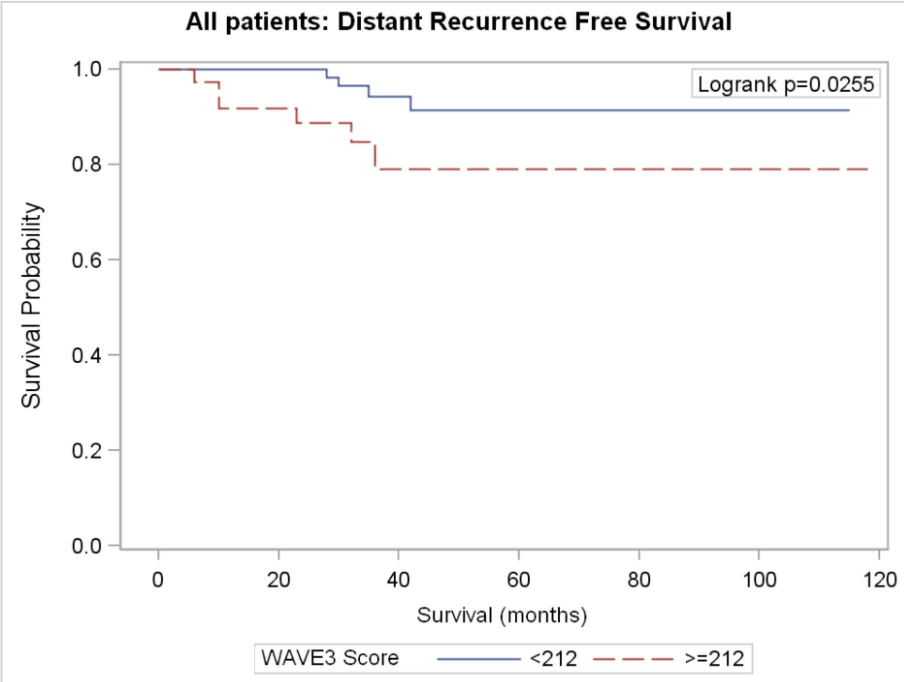
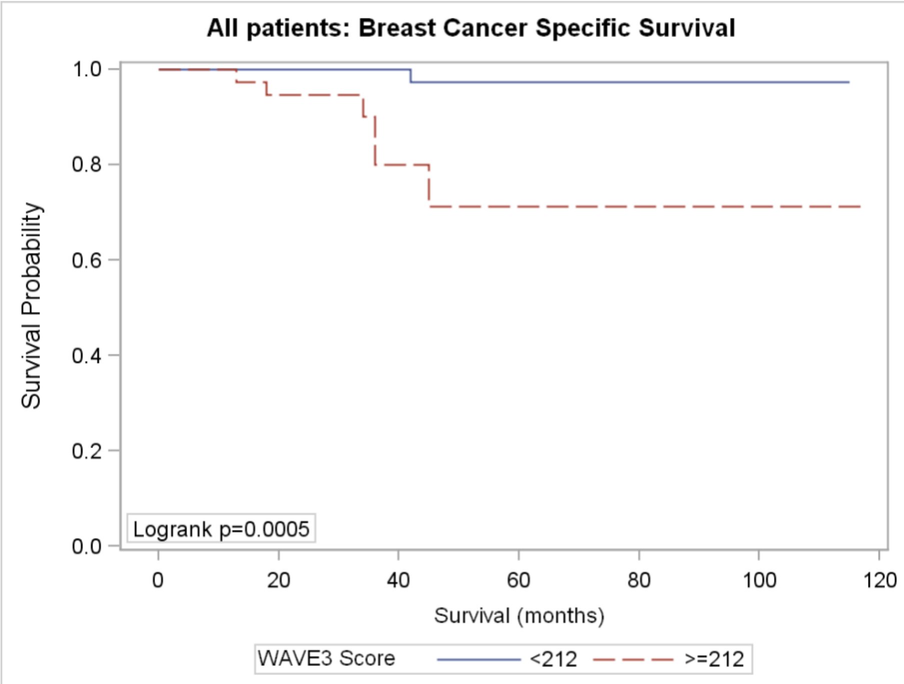


Figure 1. WAVE3 score ≥212 was significantly associated with decreased distant recurrence free survival in the entire study group and the SBR3/ER- cohort (p=0.0255 and p=0.0156, respectively).

1.B.2. The recurrence free survival is tumor grade-dependent.

We also found that a very significant correlation between the tumor grade and the overall disease free survival. Patients with grade 1 tumors and low WAVE3 staining tend to live longer with no detectable disease compared to those patients with grade 3 tumors and high levels of WAVE3 staining.



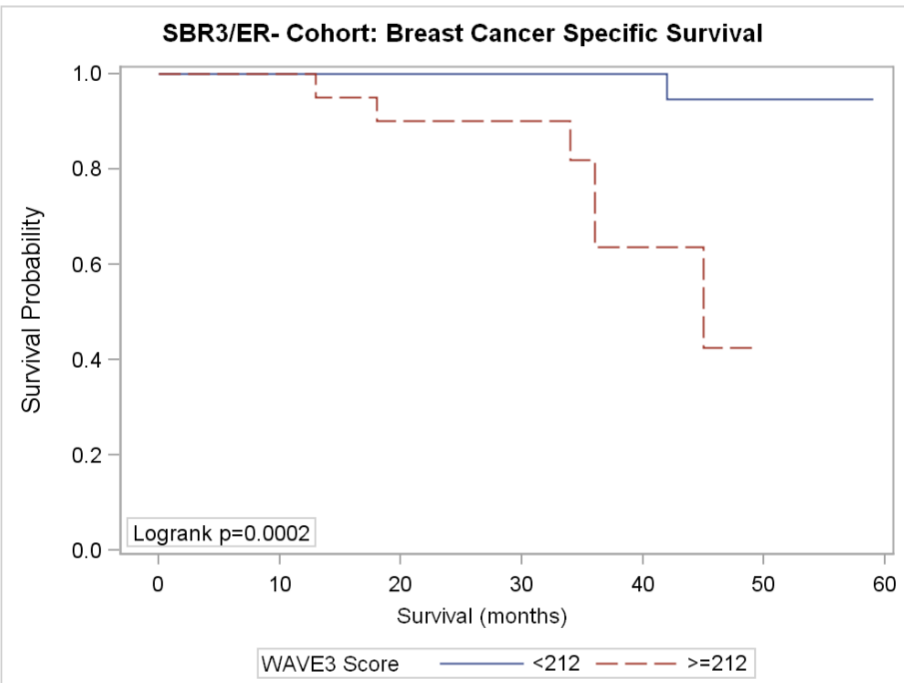


Figure 2. WAVE3 score ≥212 was significantly associated with decreased breast cancer specific survival in the entire study group and the SBR3/ER- cohort (p=0.0005 and p=0.0002, respectively

On multivariate analysis a WAVE3 score ≥212 did not remain independently associated with distant disease free survival (p=0.062) but was independently associated with an increased risk for breast cancer specific mortality (p=0.009).

1.B.3. Correlation between the WAVE3 staining levels and distant metastasis.

One of the very significant finding is that WAVE3 staining levels can be used an independent marker for survival recurrence as well as for disease-specific mortality risk.

1.B.4. CONCLUSIONS.

An association between ER status, SBR grade and WAVE3 score was not verified. WAVE3 score is associated with tumor size, lymph node status, and pathologic stage. Patients in the SBR3/ER- group were more likely to develop distant recurrence and disease specific mortality. A WAVE3 score $\geq$ 212 was associated with breast cancer specific survival on uni- and multivariate analysis. WAVE3 score may be able to predict adverse outcome in high risk breast cancer patients.

Specific Aim 2: Evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells after the administration of adjuvant chemotherapy in women with operable breast cancer.

2.A. Performance of Tasks proposed in the SOW

Task 5: We proposed to identify the blood specimens for which the most complete and informative clinical and pathological and outcome data is available.
We will analyze 100 samples from Stage I and/or II breast cancer patients, and 100 samples from healthy females without cancer history.

We have identified all the samples described above which were linked to complete clinical and pathological and outcome data. The blood samples were shopped to Cleveland Clinic about three months ago.

Task 6: We proposed to:
Determine the presence or absence of WAVE3 mRNA in the blood of breast cancer patients before and after chemotherapy. The blood from subjects with no evidence of disease will be used as controls. We will:

a) Prepare total RNA from the blood specimens using standard RNA extraction protocols.
b) Assess the quality of the RNA by RNA-Agarose gel electrophoresis.
c) Use β–Actin and other internal controls such as WAVE2 (WAVE2 is ubiquitously expressed in the white blood cells) to ensure the quality of the RT-PCR and as loading controls.
d) Perform Nested RT-PCR and agarose gel electrophoresis to determine the presence or absence of WAVE3 transcripts in the blood specimens, and record the results.
e) Repeat tasks b to d at least 3 times to insure reproducibility of the results.
f) Repeat tasks a to e for the specimens with questionable results.

Completed. See below

Task 7: A BC TMA is being Built that contains more than 120 BC specimens from different stages and genetic subtype.

Task 8: Underway

2.B. Background.

WAVE3 is expressed at very low levels in the hematopoietic cells, but its expression levels are higher in the circulating tumor cells (CTC) as a result of early tumor cell dissemination even in patients with small tumors. We used this WAVE3-specific characteristic to evaluate the prognostic value of WAVE3 expression levels in CTCs in women with operable breast cancer. A second cohort consisted of 200 BC patients from whom blood was collected before surgery and initiation of therapy. Quantitative real-time RT-PCR was utilized to determine the expression levels of WAVE3 in total RNA extracted from the circulating tumor cells. The WAVE3 score between each subtype was compared and the association between WAVE3 score and a variety of clinico-pathologic features was examined

2.C. Methods.

Total RNA was extracted from each sample's Buffy coat using TRIzol reagent (Invitrogen), following to the manufacturer's instructions. cDNA was generated and used as a template for semi-quantitative RT-PCR performed as previously described (5;8;14;15). Expression levels of microRNAs were quantified by real-time quantitative RT-PCR using the human TaqMan MicroRNA Assays Kits (Applied Biosystems). The reverse transcription reaction was carried out with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. For each gene, quantification of expression levels was performed using the respective gene-specific primers (Table S2) and the RT^2 SYBR Green/Fluorescein qPCR Master Mix (SABiosciences) following the manufacturer's instructions. Quantitative PCR was performed on the BioRad iCycler PCR system where the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The cycle threshold (Ct) values were calculated with SDS 1.4 software (Bio-Rad). The expression levels of each transcript were normalized using the $2^{-\Delta\Delta Ct}$ method (16) relative to GAPDH. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the transcript of interest. The ΔΔCt was then calculated by subtracting ΔCt of the matching normal human breast tissue from the ΔCt of cancer tissues, or the ΔCt of MCF10A cell line for the established cancer cell lines. Fold change in the gene was calculated according to the equation $2^{-\Delta\Delta Ct}$.

The WAVE3 expression levels (score) between each cohort was compared and the association between WAVE3 score and a variety of clinico-pathologic features was examined. The log rank test was used to compare distant recurrence free survival at various WAVE3 scores. A score of ≥212 was found to have the strongest association with distant recurrence and was used as a positive threshold for subsequent survival analyses.

Analysis of categorical data between two groups was performed with the χ^2 square test. Analysis of continuous variables for two groups was with the Mann-Whitney rank sum or the Pearson product moment correlation. Analysis of continuous variables for more than two groups was with the Kruskal-Wallis one way ANOVA . Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. The multiple linear regression method was performed to compare survival as a function of patient and treatment factors.

2.D. Results

2.D.1. WAVE3 transcripts can easily be detected in the blood of metastatic breast cancer patients and not in the normal blood.

We have conducted a pilot study as a proof of principle to determine whether WAVE3 can be detected in the blood of metastatic cancer patients. We randomly chose 10 blood samples (from metastatic breast cancer patients) that are part of the archived blood repository, which were matched with 10 blood samples from healthy controls without any cancer history. The Nested-RT-PCR assay showed that while no WAVE3 mRNA could not be detected in any of the control blood samples without cancer history, different levels of WAVE3 mRNA was amplified from all six blood specimens belonging to patients with metastatic breast cancer (Figure 1).

These preliminary data, while they confirm the sensitivity of our assay (Figure 1), also provide a preliminary demonstration that WAVE3 could be used as a biomarker for early follow-up on the progression and relapse of metastatic cancer.

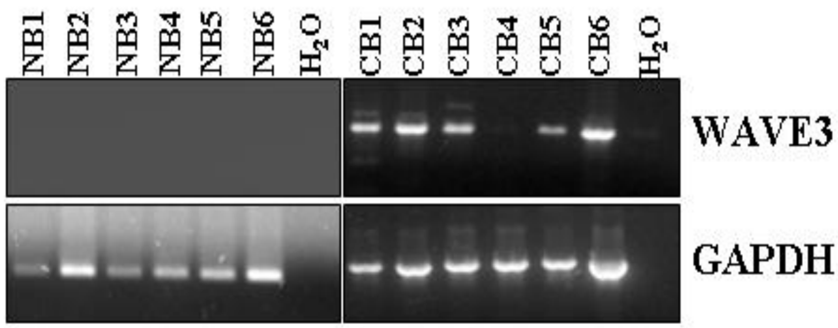


Figure 1: Nested RT-PCR from total RNA extracted from blood cells of six healthy control individuals and from the blood cells of six randomly chosen patients with metastatic cancer. WAVE3 mRNA could be amplified from the breast cancer patients, but not form from the healthy controls. GAPDH was used as an internal control for the integrity of the RNA and as an equal loading control.

2.D.2. COHORT CHARACTERISTICS

Variable	N	N Missing	Min	25%	Median	75%	Max	Mean	Std
WAVE3.RT.Score	199	1	0.5	10.3	23.54	43.39	99.54	29.84	23.57
Age At Draw	200	0	23	43	53	62.25	88	53.56	13.35
T Size	193	7	0.01	0.9	1.5	2.3	11	1.84	1.55
Nodes Resected	199	1	1	3	6	14	42	9.48	8.95
Nodes Positive	199	1	0	0	0	1	27	1.41	3.43
Days To Recurrene	9	191	0	236	421	671	804	431	281.16
Age At Recurrence	9	191	32	35	54	56	79	51.44	16.8

Other patients chacateristics:

Variable	level	N	percentage
SampleStatusme	No prior treatment (excluding diagnostic biopsy)	159	79.5
	Post-surgical/No adjuvant or systemic therapy	41	20.5
Overnight	No	200	100
CaseControl	Case	200	100
Sex	Female	200	100
Race	Black	26	13
	White	174	87
ParticipantAttributeTypeDesc	New (Distant)	1	0.5
	New (In Situ)	5	2.5
	New (Localized)	128	64
	New (Regiol)	66	33
FilSurg	Lumpectomy	131	65.5
	Total mast w/immediate reconstruct	32	16
	Total mast w/no immediate reconstruct	37	18.5
AxilStage	Axil dissection level I/II (lumpectomy or MRM)	66	33.2
	Axil dissection level I/II/III	1	0.5
	Sentinel Node biopsy only	132	66.3
SizeNotation	Multicentric	1	3.6
	Multifocal	8	28.6
	Neoadjuvant	18	64.3
	Not reported	1	3.6

ClinicalStg	I T1 N0 M0	1	5.6
	IIA T1 N1 M0	1	5.6
	IIA T2 N0 M0	6	33.3
	IIB T2 N1 M0	4	22.2
	IIB T3 N0 M0	1	5.6
	IIIA T3 N1 M0	1	5.6
	IIIB T4 Any N M0	2	11.1
	IIIB T4 N1 M0	2	11.1
Stage	I T1 N0 M0	94	47
	IIA T1 N1 M0	25	12.5
	IIA T2 N0 M0	31	15.5
	IIB T2 N1 M0	20	10
	IIB T3 N0 M0	2	1
	IIIA T1 N2 M0	5	2.5
	IIIA T2 N2 M0	8	4
	IIIA T3 N1 M0	4	2
	IIIA T3 N2 M0	1	0.5
	IIIB T4 Any N M0	2	1
	IIIB T4 N1 M0	2	1
	IIIC Any T N3 M0	6	3
ER	Negative	96	48
	Positive	104	52
PR	Negative	101	50.5
	Positive	99	49.5
Her.2	Negative	166	83.8
	Strong	31	15.7
	Weak	1	0.5
NuclearGrade	I	22	11.7
	II	56	29.8
	III	110	58.5
VascLymphaInvas	No	132	72.9
	Yes	49	27.1
Necrosis	No	135	71.8
	Yes	53	28.2
Bilateral	FALSE	190	95
	TRUE	10	5
MenopausalStat	"2 = Yes, menstrual periods on hormone replacement therapy"	2	1
	tural Periods	75	37.5
	No Periods	123	61.5
ClinT	T1	1	0.5
	T1a	1	0.5
	T1b	39	19.5
	T1c	70	35
	T2	49	24.5
	T3	8	4
	T4d	4	2
	Tis	9	4.5
	Tx	19	9.5
ClinN	N0	171	85.5
	N1	22	11
	N2	2	1
	Nx	5	2.5
PathT	T1a	16	8.7

	T1b	32	17.5
	T1c	73	39.9
	T1mic	3	1.6
	T2	51	27.9
	T3	6	3.3
	T4d	1	0.5
	Tx	1	0.5
PathN	pN0	116	63.4
	pN0(i+)	1	0.5
	pN0(i-)	2	1.1
	pN1a	35	19.1
	pN1c	1	0.5
	pN1mi	7	3.8
	pN2a	15	8.2
	pN3	2	1.1
	pN3a	4	2.2
Grade	I (well diff.)	8	4.2
	II (mod. diff.)	36	18.8
	III (poorly diff.)	147	77
Histology	Ductal clinical inflammatory	2	1
	Ductal inflammatory (w/path dermal lymph invasion)	1	0.5
	Ductal invasive, NOS	125	62.5
	Ductal papillary	1	0.5
	Invasive Mixed Ductal and Lobular	3	1.5
	Invasive w/predomint intraductal component	1	0.5
	Invasive with predomint intraductal component	48	24
	Lobular invasive	18	9
	Metaplastic	1	0.5
FirstRecurSite	Bone	1	11.1
	Ipsilateral breast	4	44.4
	Liver, parenchyma	2	22.2
	Skin	2	22.2
X1stRecurTx	Arimidex	1	12.5
	Bevacizumab (Avastin)	1	12.5
	Capecitabine	2	25
	Gemcitabine-Albumin-bound Paclitaxel (Abraxane)	1	12.5
	Gemcitabine-Capecitabine	1	12.5
	Zolendrote (Zometa)	1	12.5
	Zometa-Lapitinib	1	12.5
ChemoType	Doxorubicin (Adriamycin, Adriamycin-TM)	1	0.8
	AC	3	2.3
	AC-Docetaxel-Herceptin	1	0.8
	AC-Herceptin	1	0.8
	AC-Paclitaxel	7	5.5
	AC-Paclitaxel-Bevacizumab (Avastin)	1	0.8
	AC-Paclitaxel-Herceptin	7	5.5
	AC-Taxol-Herceptin	1	0.8
	AC-Taxotere	1	0.8
	CT (Cyclophosphamide(Cytoxan)/Paclitaxel (Taxol)	2	1.6
	Clinical Trial Drug--nonblinded	1	0.8
	Cyclophosphamide (Cytoxan, CTX)-Albumin-	1	0.8

	bound Paclitaxel (Abraxane)		
	Cyclophosphamide(Cytoxan)/Docetaxel(Taxotere)	13	10.2
	Cyclophosphamide(Cytoxan)/Docetaxel(Taxotere)-Clodrote	1	0.8
	Cyclophosphamide(Cytoxan)/Docetaxel(Taxotere)-Herceptin	2	1.6
	Docetaxel (Taxotere)-Carboplatin	1	0.8
	Docetaxel (Taxotere)-Herceptin	1	0.8
	Docetaxel (Taxotere)-Liposomal Doxorubicin (Doxil)	1	0.8
	Docetaxel (Taxotere)-carboplatin-Herceptin	1	0.8
	Dose-dense AC followed by Paclitaxel (Taxol)	54	42.2
	Dose-dense AC followed by Paclitaxel (Taxol)-Albumin-bound Paclitaxel (Abraxane)	3	2.3
	Dose-dense AC followed by Paclitaxel (Taxol)-BlindedDrugTrial	3	2.3
	Dose-dense AC followed by Paclitaxel (Taxol)-Cytoxan-Taxotere	1	0.8
	Dose-dense AC followed by Paclitaxel (Taxol)-Herceptin	10	7.8
	Dose-dense AC followed by Paclitaxel (Taxol)-Herceptin-Taxotere	1	0.8
	Dose-dense AC followed by Paclitaxel (Taxol)-Zoladex	1	0.8
	Doxorubicin(Adriamycin)/Docetaxel(Taxotere)	1	0.8
	Doxorubicin(Adriamycin)/Docetaxel(Taxotere)-Taxol	1	0.8
	Doxorubicin(Adriamycin)/Paclitaxel(Taxol)-Herceptin	1	0.8
	Other GnRH agonist	1	0.8
	Paclitaxel (Taxol)-Herceptin	1	0.8
	TAC	1	0.8
	Trastuzumab (herceptin, anti-HER2mab)	2	1.6
SBR	1	41	20.5
	2	60	30
	3	99	49.5

2.D.3. Association between WAVE3 expression levels and cohorts paramaters

Variable	**SBR1/ER+**	**SBR3/ER-**	***p* value***
	Mean (SD)	**Mean (SD)**	
Age at Diagnosis (years)	**51.33 (± 12.15)**	**54.86 (± 14.8)**	**0.172**
Tumor Size (cm)	**1.36 (± 1.07)**	**2.27 (± 1.99)**	**0.002**
	n (%)	**n (%)**	
Lymph Node Status**			**0.736**
Negative	**25 (62.5)**	**43 (57.3)**	
Positive	**15 (± 37.5)**	**32 (42.7)**	
TNM Stage			**0.009**
Stage I	**31 (81.6)**	**37 (50)**	
Stage II	**7 (18.4)**	**29 (39.2)**	
Stage III	**0 (0)**	**5 (6.8)**	
Stage IV	**0 (0)**	**3 (4.1)**	
Her2 Status			**0.01**
Negative	**39 (97.5)**	**58 (77.3)**	
Positive	**1 (2.5)**	**17 (22.7)**	
Histology			**0.144**
Ductal	**24 (60)**	**57 (75)**	
Invasive	**16 (40)**	**19 (25)**	
Grade			**<0.00001**
I (well diff.)	**7 (18.9)**	**19 (25)**	
II (mod. diff.)	**14 (37.8)**	**2 (2.7)**	
III (poorly diff.)	**16 (43.2)**	**71 (97.3)**	

Figure 1. WAVE3 expression levels in CTCs are associated with ER-negative BC

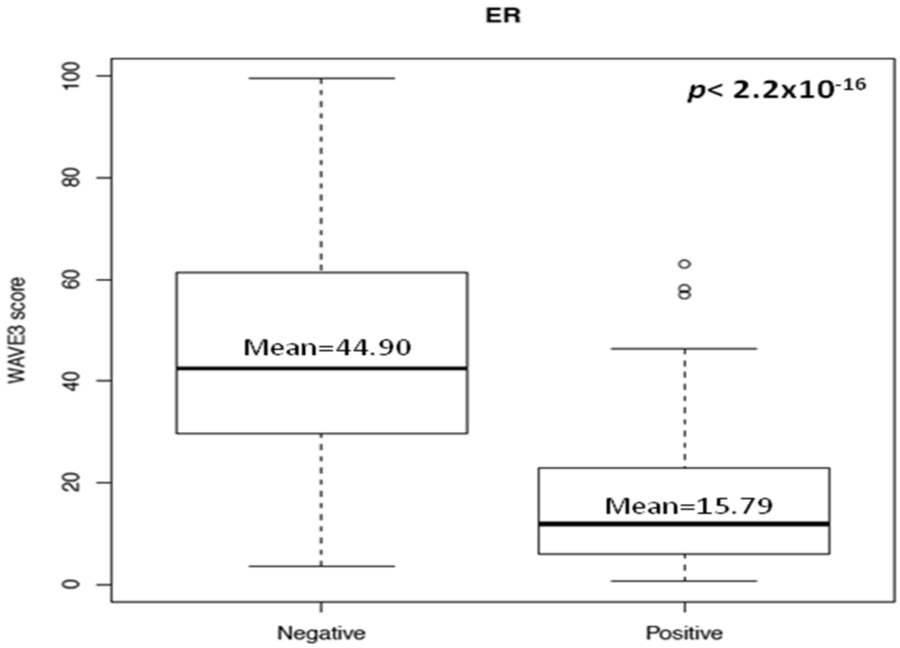


Figure 2. WAVE3 expression levels in CTCs are associated with PR-negative BC

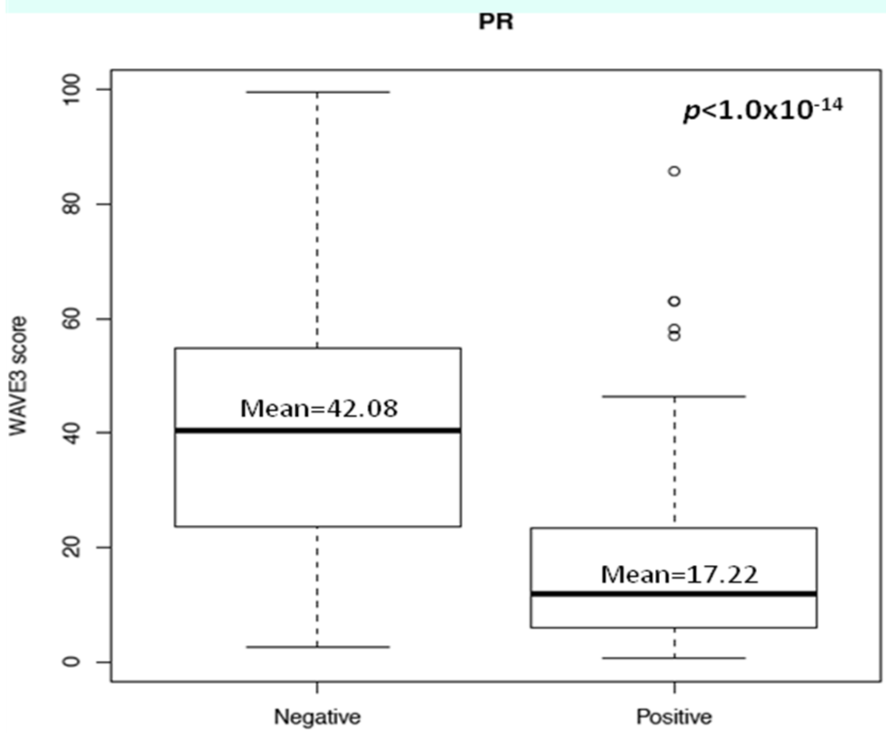


Figure 3. WAVE3 expression levels in CTCs are associated with tumor size in BC

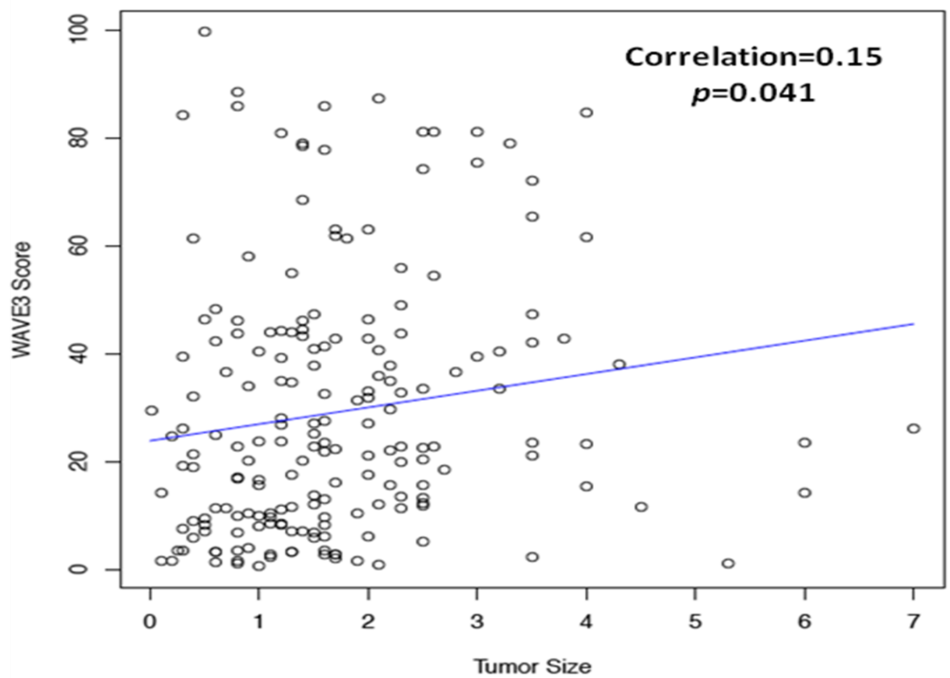
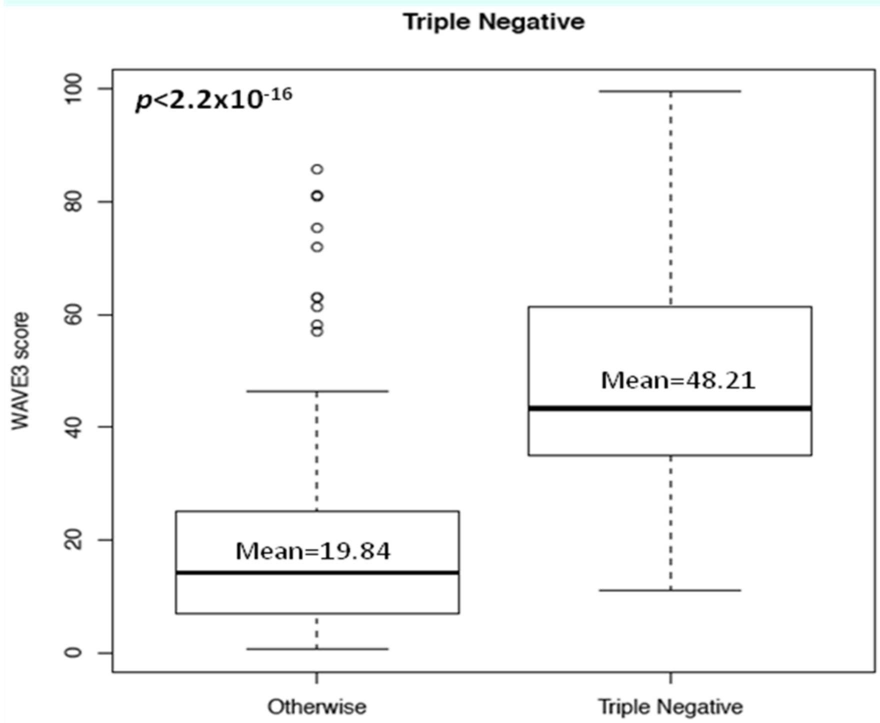


Figure 4. WAVE3 expression levels in CTCs are associated with triple-negative BC



The major finding from Aim2 is that WAVE3 expression levels in the CTCs are significantly associated with the triple-negative tumors and may be used as a marker for this BC subtype.

KEY RESEARCH ACCOMPLISHMENTS

Major conclusions from this study are:

Specific Aim 1: To test whether WAVE3 levels can be used as a predictive marker for the progression and metastasis of breast cancer.

The major findings from the WAVE3 staining in the primary breast cancer tumors are as follows:

1. WAVE3 staining correlates with tumor grade.
   We found a significant correlation between the tumor grade and WAVE3 staining levels. Low grade tumors show low WAVE3 score versus the high grade tumors which showed high WAVE3 score.

2. Low levels of WAVE3 staining correlate with reduced Distant Recurrence or Breast Cancer Related Mortality.
   We found a very significant correlation between the levels of WAVE3 staining in the primary tumors and the overall disease free survival. Those patients with low levels of WAVE3 staining in the primary tumors were found to live longer disease free after the primary tumors was removed compared to those patients with high levels of WAVE3 staining.

3. **The recurrence free survival is tumor grade-dependent.**
   We also found that a very significant correlation between the tumor grade and the overall disease free survival. Patients with grade 1 tumors and low WAVE3 staining tend to live longer with no detectable disease compared to those patients with grade 3 tumors and high levels of WAVE3 staining.

Specific Aim 2: To evaluate the prognostic value of WAVE3 expression levels in circulating tumor cells after the administration of adjuvant chemotherapy in women with operable breast cancer.

The major finding from the evaluation of WAVE3 expression levels in the blood of breast cancer patients is as follows.

4. **WAVE3 expression levels in the CTCs are significantly associated with the triple-negative tumors and may be used as a marker for this BC subtype.**

REPORTABLE OUTCOMES

Based on these findings and on the findings from ongoing research projects dealing with different aspects of the involvement of WAVE3 in the biology and etiology of BC, we were able to publish the following:

Manuscripts in peer-reviewed scientific journals:

1-Taylor MA, **Sossey-Alaoui K,** Thompson C, Danielpour D, Schiemann WP. TGF-beta upregulates miR-181a expression to promote breast cancer metastasis. **J Clin Invest.** December 2012. **In Press.** PMID: 23241956.

2-Sossey-Alaoui K. Surfing the big WAVE: Insights into the role of WAVE3 as a driving force in cancer progression and metastasis. **Semin Cell Dev Biol.** 2012 Oct 29. 9521(12)00186-3.

3-Kulkarni S, Augoff K, Rivera L, McCue B, Khoury T, Groman A, Zhang L, Tian L, **Sossey-Alaoui K**. Increased expression levels of WAVE3 are associated with the progression and metastasis of triple negative breast cancer. **PLoS One**. 2012;7(8):e42895.

4-Augoff K, McCue B, Plow EF, **Sossey-Alaoui K**. miR-31 and its host gene lncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer. **Mol Cancer.** 2012 Jan 30;11:5

5-Augoff K, Das M, Bialkowska K, McCue B, Plow EF, **Sossey-Alaoui K**. miR-31 is a broad regulator of β1-integrin expression and function in cancer cells. **Mol Cancer Res.** 2011 Nov;9(11):1500-8.

6-Sossey-Alaoui K, Downs-Kelly E, Das M, Izem L, Tubbs R, Plow EF. WAVE3, an actin remodeling protein, is regulated by the metastasis suppressor microRNA, miR-31, during the invasion-metastasis cascade. **Int J Cancer**. 2011 Sep 15;129(6):1331-43.

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1-Sossey-Alaoui K. The WAVE3-dependent cancer cell invasion is regulated by miR-31 during cancer progression and metastasis. Proceedings of the 101st Annual Meeting of the AACR. Abstract #2022, 2010.

2-Khalid Sossey-Alaoui, Erinn Downs-Kelly, Mitali Das, Lahoucine Izem, Raymond Tubbs and Edward F. Plow. The WAVE3-dependent cancer cell invasion is regulated by miR-31 during cancer progression and metastasis. Proceedings of Research Day of the Lerner Research Institute. Cleveland Clinic. 2010.

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4-Augoff K, Zhang L, Rivera L, Khoury T, Tian L, Groman A, Watroba N, Plow EF, Kulkarni S, **Sossey-Alaoui K.** Increased expression levels of WAVE3 are associated with breast cancer progression and metastasis. Proceedings of the 102nd Annual Meeting of the AACR. Abstract # 3206, 2011.

5-Sossey-Alaoui K, Kulkarni S, Khoury T, Tian L, Zhang L, Augoff K, Plow EF. WAVE3 expression levels are associated with breast cancer progression and metastasis. Department of

Defense Breast Cancer Research Program Era of Hope Meeting. Abstract # BC073783-2636, 2011.

6- Taylor MA, Davuluri G, Schiemann WP, **Sossey-Alaoui K.** WAVE3 is required for TGF-β-mediated EMT in Breast Cancer. AACR special conference on *Tumor Invasion and Metastasis*. Abstract #79962_1. 2013.

CONCLUSION.

The major conclusions from this study so far are:

- WAVE3 score is positively correlated with tumor size, lymph node status and pathologic stage.
- Only patients in the SBR3/ER- cohort experienced distant recurrence or disease specific mortality. WAVE3 scores were higher in patients with adverse clinical outcome.
- A WAVE3 score $\geq$ 212 was associated with breast cancer specific survival on uni- and multivariate analysis.
- WAVE3 score is independently associated with an increased risk for breast cancer specific mortality
- WAVE3 score may be able to predict adverse outcome in high risk breast cancer patients.
- WAVE3 expression levels in the CTCs are significantly associated with the triple-negative tumors and may be used as a marker for this BC subtype.

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Appendices: None